Appendix D

(D1 – D6)

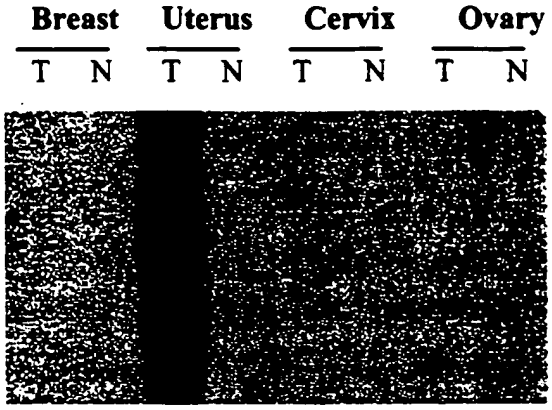
Appendix D1

Northern Blot Analysis

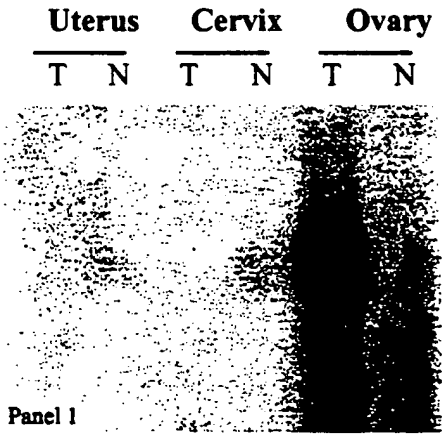
Total RNA from several cancer cell (*e.g.*, breats, uterus, cervix, ovary, all of which are commercially available) and cell lines (*e.g.*, RIN-5AH, HEP-G2, A549, HELA, MOLT4, HL-60 and SW480, all of which are commercially available) were isolated using TRIzol reagent (Gibco/BRL, Cat #15596-018) according to manufacturer's instructions. After electrophoreseis in a 1% agarose/formaldehyde gel, the RNA was transferred to a nylon membrane using standard protocols. A ^{32}P-labelled GPCR probe was synthesized using a DNA fragment corresponding precisely to the entire coding sequence and a Prime It II Random Primer Labeling Kit (Stratagene, Cat. #300385) according to manufacturer's instructions. Hybridization was performed using ExpressHyb Solution (Clontech, Cat.#8015-2) supplemented with 100ug/ml salmon sperm DNA as follows. The membrane containing the separated RNA samples were first incubated with ExpressHyb solution at 65°C for 1 hour. The ^{32}P-labeled GPCR DNA probe was denatured by boiling for 2 min, placed on ice for 5 min and then transferred into the ExpressHyb solution bathing the membrane. After an overnight incubation at 65°C, the membrane was removed from the hybridization and washed four times for 15 min each in 2XSSC/1% SDS at 65°C, followed by two washes for 15 min each in 0.1XSSC/0.5% SDS at 55°C. Excess moisture was removed from the blot by gentle shaking, after which the blot was wrapped in plastic and exposed to film overnight at -80°C.

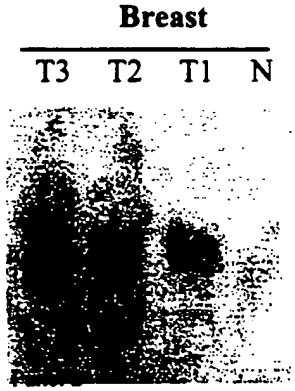
Appendix D

D2
(19Y)

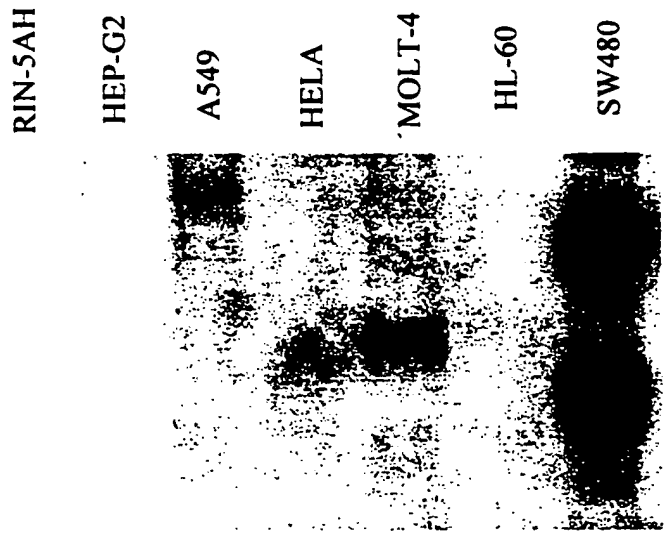


D3
(18A)





D4
(18AI)



Appendix D5

Proliferation Assay Protocol

1. Expression of 19Y in Prostate Cancer Cells Protocol

To test the effect of 19Y on cellular proliferation *in vitro*, the prostate cancer cell line PC-3 was transfected with a CMV-19Y expression plasmid using Lipofectamine (Gibco/BRL). Cells were then incubated in the presence of 500 μg/ml G418 in order to generate pools of PC-3 cells that expressed 19Y in a stable manner. The effect of 19Y on PC-3 cell proliferation was then examined by incubating cells in the presence of the tetrazolium salt WST-1 and measuring its conversion to formazan. This assay (Roche Molecular Biochemicals, Cat. No. 1644807) is a standard method for quantifying cell proliferation. In brief, as cells proliferate, there is a corresponding increase in the amount of mitochondrial dehydrogenase activity. WST-1 is a slightly red tetrazolium salt that is converted by these dehydrogenases to formazan, a dark red dye whose formation is easily measurable by colorimetric methods. Thus, cell proliferation can be quantitated by incubating cells in the presence of WST-1 and measuring the formation of formazan.

2. WST Proliferation Assay Protocol

In brief, the expriment was performed as follows. $5X10^4$ cells (PC-3/CMV or PC-3/19Y) were seeded in each well of a 96-well microtiter plate and incubated for 24 hr. In some wells, the cells were incubated with inverse agonists directly identified using constitutively active 19Y, at a concentration of 10μM compound. After the incubation period, 10μl of the Cell Proliferation Reagent WST-1 (Roche Molecular Biochemicals) was added to each well. The cells were then incubated for an additional 0.5 to 4 hr. Finally, the formation of formazan was measured in a microtiter plate reader at a wavelength of 440 nm.

Appendix D6

PC-3 cell proliferation is induced by 19Y and reversed by 19Y-selective inverse agonists